21. Neonatal mice (C57BL/6, postnatal day 0-1) were anesthetized with ketamine (>30 mg/kg, intraperitoneally), and then killed by cervical dislocation. The experimental procedures met the regulation of the Animal Care Committee of Osaka University Graduate School of Medicine. Neurons were cultured at low density by using conventional methods (*38*). A piece of visual cortex was removed from neonatal mice, enzymatically dissociated with papain (20 U/ml), and triturated with a fire-polished glass pipette. Neurons were plated on previously prepared glial feeder layer, and were grown in a solution based on Neurobasal A Medium (Gibco, Rockville, MD) supplemented with 5% B27 (Gibco). All experiments were carried out 14 to 24 days after plating.
22. The cDNA of mouse BDNF tagged with GFP at the COOH-terminus was provided by M. Kojima (*20*). Glass micropipettes were filled with TE buffer (pH 8.0), which contained cDNA of BDNF-GFP (0.5 to 1 μg/μl) or both cDNAs of BDNF-GFP (0.5 μg/μl) and DsRed (0.5 μg/μl, pDsred-N1; Clontech, Palo Alto, CA). Cultured neurons were placed on the stage of an inverted epifluorescence microscope (TE300; Nikon, Tokyo, Japan), and cDNAs were injected into the nucleus of a neuron through a micropipette under visual control, using a micromanipulator (MMO-202ND; Narishige, Tokyo, Japan).
23. Neurons that expressed BDNF-GFP and DsRed signals were observed with a 40×, 1.3 NA oil immersion objective (Nikon) attached to the inverted epifluorescence microscope. The fluorescence of GFP, DsRed, aminomethylcoumarin (AMCA), and Cy5, excited by light at the wavelength of 480, 530, 350, and 650 nm, respectively, was measured using a cooled charge-coupled device (CCD) camera (C4742-95; Hamamatsu Photonics, Hamamatsu, Japan). This system consisted of 1024 pixels by 1024 pixels, each of which corresponded to 0.17 μm by 0.17 μm with the 40× objective. Data were further analyzed with an Aquacosmos system (Hamamatsu Photonics). Neurons were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) and 4% sucrose in Dulbecco's phosphate-buffered saline (PBS) for 20 or 40 min at room temperature. The cells were incubated with PBS containing 0.2% Triton-X (Sigma) for 1 min and were blocked by 10% donkey serum in PBS for 1 hour at 37°C. Then, monoclonal antibody to MAP2 (1:250, Sigma), monoclonal antibody to tau (1:150; Cedarlane, Hornby, Ontario, Canada), polyclonal antibody to BDNF (2 μg/ml, provided by R. Katoh-Semba), polyclonal antibody to synapsin I (1:500; Chemicon, Temecula, CA) or polyclonal antibody to TrkB (1:100; Santa Cruz, Santa Cruz, CA) was applied for 2 hours at 37°C. Synapsin I, TrkB and endogenous BDNF were visualized by secondary antibody to rabbit conjugated with Alexa 546 (1:2000; Molecular Probe, Eugene, OR) or Cy5 (1:200; Chemicon). MAP2 and tau were visualized by secondary antibody to mouse conjugated with Alexa 546 or AMCA (1:100, Chemicon).
24. Web figure 1 and Web movie 1 are available at *Science* Online at www.sciencemag.org/cgi/content/full/291/5512/2419/DC1.
25. W. Haubensak, F. Narz, R. Heumann, V. Lessmann, *J. Cell Sci.* **111**, 1483 (1998).
26. In three neurons, synaptic and action currents were recorded through a whole-cell patch electrode (resistance, 3 to 5 megohm) in a voltage-clamp mode with a patch-clamp amplifier (Axoclamp 2B; Axon Instruments, Foster, CA) 48 hours after the intranuclear injection of cDNAs. The signal was digitized at 10 kHz and filtered at 2 kHz. Recordings were carried out at room temperature. The perfusing solution, unless otherwise noted, contained the following: NaCl, 120 mM; KCl, 4 mM; $CaCl_2$, 2 mM; $MgSO_4$, 1 mM; Hepees, 10 mM; glucose, 10 mM(pH 7.4). Osmolality was adjusted to 250 to 255 mOsm by adding sucrose when necessary. The electrode solution was as follows: K-gluconate, 110 mM; Hepes, 10 mM; KCl, 10 mM; EGTA, 0.5 mM; MgATP, 5 mM; and Na_2GTP, 1 mM.
27. A. Matus, *Annu. Rev. Neurosci.* **11**, 29 (1988).
28. Time-lapse recordings were carried out 16 to 24 hours after transfection and sequential images were acquired with a cooled CCD camera at room temperature. Exposure time was 1 or 2 s, lapse of time was 3 or 5 s, and the total time was 10 to 20 min. The solution for the time-lapse recordings contained the following: NaCl, 120 mM; KCl, 4 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2 mM; $MgSO_4$, 1 mM; Hepes, 20 mM; glucose, 30 mM (pH 7.4).
29. T. Nakata, S. Terada, N. Hirokawa, *J. Cell Biol.* **140**, 659 (1998).
30. Localization of synapsin I in DsRed-positive puncta representing presynaptic sites was visualized in 10 neurons with immunocytochemistry, as mentioned (*23*).
31. A quantitative assessment of fluorescence intensity was carried out 48 hours after the intranuclear injection of cDNA. BDNF-GFP was visualized by polyclonal antibody to GFP (1:1000, Molecular Probe) or monoclonal antibody to GFP (1:500, Molecular Probe) and secondary antibody to rabbit or mouse conjugated with Cy5, to avoid autofluorescence of neurons and glia at 480 nm. The fluorescence intensity was measured on a square window (30 pixels by 30 pixels) placed on a soma which contacted DsRed-positive axon terminals, and the mean fluorescence intensity of 900 pixels was calculated. As control, another soma that did not contact DsRed-positive terminals was randomly selected from the same culture dish, and the intensity of background fluorescence was calculated as above.
32. TrkB-IgG fusion protein (Genentech, San Francisco, CA) was applied to neurons after transfection through the medium at 40 μg/ml, which is known to block function of TrkB ligands (*39*).
33. TTX (Wako Pure Chemicals, Osaka, Japan) and picrotoxin (Tocris Cookson, Bristol, UK) were applied to neurons through the perfusion medium at the concentration 50 and 1 μM, respectively. To confirm that these drugs actually affect activities of neurons, synaptic and action potentials were observed for 5 min each before and after the application. In some experiments, picrotoxin or TTX was applied to neurons for 48 hours after the intranuclear injection of cDNAs. Synaptic and action potentials were recorded in the current-clamp mode in the same way as described (*26*) except for the clamp mode.
34. C. S. von Bartheld, M. R. Byers, R. Williams, M. Bothwell, *Nature* **379**, 830 (1996).
35. L. J. Goodman *et al.*, *Mol. Cell. Neurosci.* **7**, 222 (1996).
36. O. Griesbeck *et al.*, *Microsc. Res. Tech.* **45**, 262 (1999).
37. A. Balkowiec, D. M. Katz, *J. Neurosci.* **20**, 7417 (2000).
38. Y. Otsu, F. Kimura, T. Tsumoto, *J. Neurophysiol.* **74**, 2437 (1995).
39. Y. Akaneya, T. Tsumoto, S. Kinoshita, H. Hatanaka, *J. Neurosci.* **17**, 6707 (1997)
40. We would like to thank M. Kojima, R. Katoh-Semba, and Genentech, Inc., for providing cDNA of BDNF-GFP, antibody to BDNF, and TrkB-IgG, respectively. We also thank T. Tachibana and F. Kimura for technical advice on intranuclear injection of cDNAs and on electrophysiology, respectively. Supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (C)–Advanced Brain Science Project from the Ministry of Education, Science, Sports, and Culture, Japan to T.T.

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Interference by Huntingtin and Atrophin-1 with CBP-Mediated Transcription Leading to Cellular Toxicity

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Expanded polyglutamine repeats have been proposed to cause neuronal degeneration in Huntington's disease (HD) and related disorders, through abnormal interactions with other proteins containing short polyglutamine tracts such as the transcriptional coactivator CREB binding protein, CBP. We found that CBP was depleted from its normal nuclear location and was present in polyglutamine aggregates in HD cell culture models, HD transgenic mice, and human HD postmortem brain. Expanded polyglutamine repeats specifically interfere with CBP-activated gene transcription, and overexpression of CBP rescued polyglutamine-induced neuronal toxicity. Thus, polyglutamine-mediated interference with CBP-regulated gene transcription may constitute a genetic gain of function, underlying the pathogenesis of polyglutamine disorders.

Huntington's disease (HD) and dentatorubral and pallidoluysian atrophy (DRPLA) are neurodegenerative disorders caused by polyglutamine expansions in the huntingtin and atrophin-1 proteins, respectively (*1–4*). Huntingtin with expanded polyglutamine aggregates in vitro and forms neuronal intranuclear and cytoplasmic inclusions in mice and in HD patients, although the inclusions themselves are not directly toxic (*5–14*). The mechanisms of polyglutamine pathogenesis remain uncertain. One hypothesis suggests that the expanded polyglutamine alters protein conformation, resulting in aberrant protein interactions (*15–17*), including interactions of the expanded polyglutamine with cellular proteins containing short polyglutamine stretches. CREB binding protein (CBP) is a coactivator for CREB-mediated transcription (*18*) and contains a 15 (mouse) or an 18 (human) glutamine stretch. CREB-mediated gene transcription promotes cell survival, and CBP is a major mediator of survival signals in mature neurons (*19–21*). CBP has been found in

polyglutamine aggregates in vitro and in vivo (*16*, *17*, *22*, *23*). We tested the hypothesis that the expanded polyglutamine in HD and related disorders can interact with the short glutamine repeat in CBP, interfering with CBP function, causing transcriptional abnormalities, and leading to cellular toxicity.

N2a neuroblastoma cells were cotransfected with constructs coding for the NH_2-terminal 63 amino acids of huntingtin with a normal or expanded repeat (Htt-N63-18Q or Htt-N63-148Q) (*24*) and a construct encoding full-length CBP (Fig. 1) (*25*). We now extend previous data (*16*, *17*) by showing that CBP (endogenous or overexpressed) is not only recruited into aggregates but also redistributed away from its normal location in the nucleus into huntingtin aggregates (Fig. 1A). Quantitation of three independent experiments where huntingtin and CBP were cotransfected showed that 86.7% (±5%) of the double transfected cells contained CBP sequestered into aggregates. Similar results were obtained with endogenous CBP. Sequestration was similar for cytoplasmic or nuclear huntingtin aggregates. The redistribution of CBP was dependent on CBP's polyglutamine stretch because CBP without the polyglutamines (CBPΔQ) was not sequestered from the nucleus (*16*, *17*) (Fig. 1B, first row). 97.3% (±1.2%) of the double transfected cells contained CBPΔQ in its normal diffuse nuclear location. CBPΔQ could be detected in some aggregates, but in contrast to CBP, CBPΔQ was not sequestered from its normal nuclear distribution. It is possible that some of the CBPΔQ could be recruited into the aggregate through interactions with other proteins or some endogenous CBP is being detected. These results show that CBP is recruited into aggregates and redistributed from its normal location through its short polyglutamine region.

The CBP homolog p300 contains a glutamine-rich region but does not contain a substantial polyglutamine repeat stretch (6Q). To determine whether mutant huntingtin could sequester p300 into aggregates, we cotransfected huntingtin with a construct encoding full-length p300 and demonstrated that expanded huntingtin did not alter p300's localization (Fig. 1B, second row). Nor was

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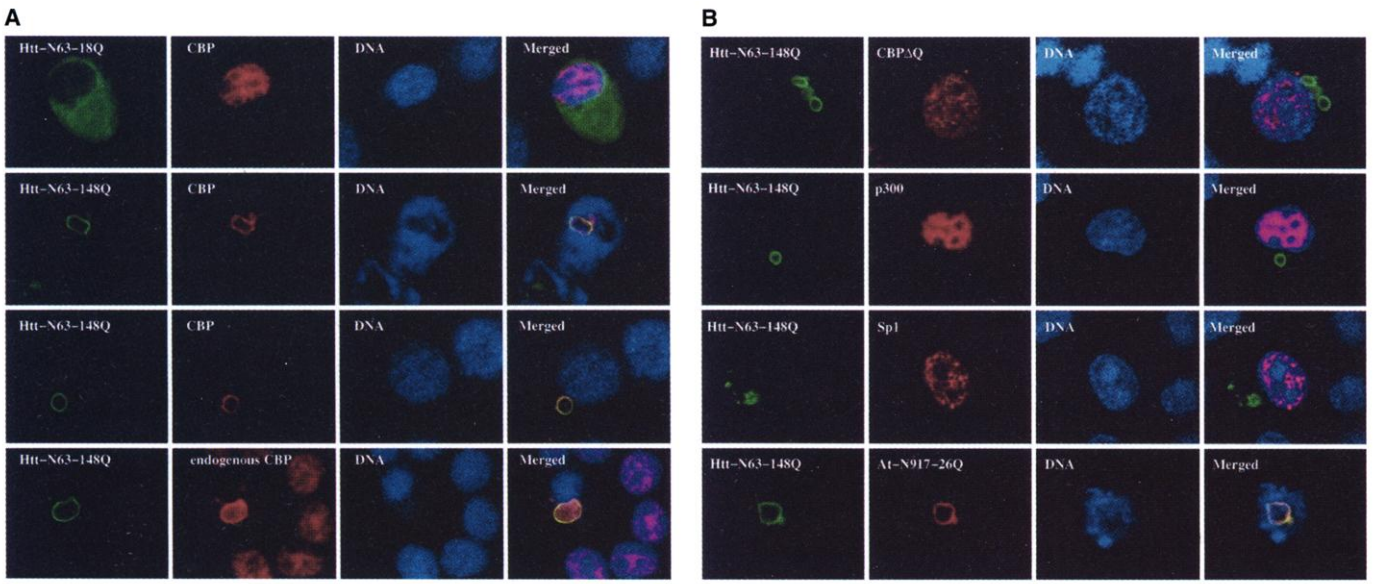

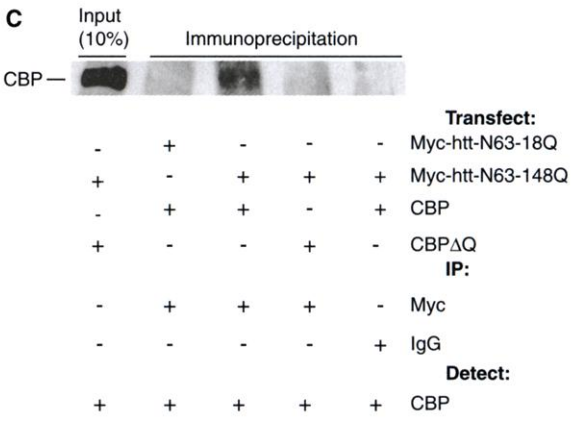

Fig. 1. Huntingtin with an expanded polyglutamine repeat sequesters CBP through direct polyglutamine interactions. (**A**) N2a cells were cotransfected with Htt-N63 (with normal or expanded repeats), and CBP (containing an 15 glutamine tract) or endogenous CBP was detected. First row: Htt-N63-18Q and CBP. Htt-N63-18Q shows diffuse cytoplasmic label, and CBP shows predominately nuclear label. These patterns are identical to those seen when each construct is transfected alone. Second and third rows: Htt-N63-148Q and CBP. Cotransfection of Htt-N63-148Q with CBP recruits CBP into the huntingtin aggregates and depletes CBP from its normal distribution in the nucleus. This effect is seen for both nuclear and cytoplasmic huntingtin aggregates. Fourth row: Htt-N63-148Q and endogenous CBP. Transfection of Htt-N63-148Q sequesters endogenous CBP into huntingtin aggregates and depletes CBP from its normal nuclear localization. (**B**) Sequestration is dependent on short polyglutamine stretches. First row: CBP with its glutamines deleted (CBPΔQ) is recruited much less into aggregates and is not depleted from its normal nuclear distribution, demonstrating the requirement of the glutamine repeat in CBP for sequestration. Second row: Htt-N63-148Q and p300 (transcription coactivator homologous to CBP, containing only six repeats). Cotransfection of Htt-N63-148Q and p300 does not sequester p300 out of the nucleus and into aggregates. Third row: Htt-N63-148Q and Sp1 (containing glutamine-rich domains, but not a glutamine repeat). When Htt-N63-148Q and Sp1 are cotransfected, there is no recruitment of Sp1 into huntingtin aggregates. Fourth row: Htt-N63-148Q and At-N917-26Q. Atrophin-1 (At-N917-26Q), another polyglutamine-containing protein (26Q), was also recruited into huntingtin aggregates. Experiments were done at least three times. (**C**) Coimmunoprecipitation: N2a cells were cotransfected with Htt-N63-18Q and CBP, Htt-N63-148Q and CBP, or Htt-N63-148Q and CBPΔQ. Antibodies for huntingtin or rabbit IgG were used for immunoprecipitation (IP). Antibodies for CBP were used for detection. Expanded huntingtin and CBP coimmunoprecipitated, suggesting a direct interaction between expanded huntingtin and CBP. Western blots of the immunoprecipitation were probed for c-myc, which recognizes the epitope-tagged huntingtin constructs and showed equal amounts of huntingtin immunoprecipitated in each lane. Experiments were repeated several times.

Sp1, a transcription factor with glutamine-rich domains, substantially recruited (Fig. 1B, third row) although it was present in some aggregates. By contrast, normal atrophin-1, which has a polyglutamine stretch (26Q), was recruited into huntingtin aggregates (Fig. 1B, fourth row).

N2a cells overexpressing Htt-N63-18Q and CBP, Htt-N63-148Q and CBP, or Htt-N63-148Q and CBPΔQ were immunoprecipitated with antibody to huntingtin and Western blotted for CBP. Htt-N63-148Q and CBP were coimmunoprecipitated, suggesting a direct interaction between expanded huntingtin and CBP (Fig. 1C). Equivalent levels of huntingtin were immunoprecipitated in these experiments, and both CBP and CBPΔQ were expressed at equal levels by Western blot analysis. Immunoprecipitation with antibody to CBP and Western blot for huntingtin showed similar results.

To determine whether CBP coaggregates with huntingtin in vivo, we performed immunocytochemistry on transgenic mice (*26, 27*) expressing an NH_2-terminal fragment of huntingtin with an expanded repeat or HD or DRPLA human postmortem brain tissue. Nuclear inclusions can be labeled with an antibody to the NH_2-terminus of huntingtin (*6, 10, 12, 26*). CBP was depleted from its normal diffusely nuclear location and sequestered into aggregates in huntingtin transgenic mice and HD and DRPLA postmortem brain tissue (Fig. 2, A and B) (*28*). In addition, CBP was also found in cytoplasmic aggregates in HD postmortem brain tissue. An antibody to p300 did not label huntingtin aggregates in transgenic mice (Fig. 2A). Similar numbers of neurons in both transgenic mice and HD patient tissue contained labeled inclusions with antibodies to either huntingtin or CBP, and most cells showed sequestration (Fig. 2, A and B).

Tissue homogenates of HD patient and control brain tissue were fractionated into pellet and high-speed supernatant fractions. CBP in the soluble fraction was analyzed by immunoprecipitation with an antibody to CBP and analyzed by Western blot. Soluble CBP was strikingly reduced in HD patient tissue compared with control brain tissue (Fig. 2C). A similar experiment with an antibody to β-tubulin showed no differences in β-tubulin, indicating that the decrease in CBP was not due to a reduction in overall protein levels. In the particulate fraction, an antibody to huntingtin yielded reactivity in a diffuse band at the top of the gel, indicating the presence of huntingtin aggregates. Comparable bands were seen when an antibody to CBP was used, indicating that CBP is found in huntingtin aggregates (Fig. 2D). Together, the fractionation data and immunohistochemistry suggest that CBP is depleted from its normal location and sequestered into aggregates.

We performed transcription assays using primary cortical neurons to determine whether interaction with mutant huntingtin alters CBP-regulated gene transcription (*29–33*). Huntingtin with a normal glutamine repeat had no effect on CBP-mediated transcription, whereas huntingtin with an expanded repeat significantly inhibited transcription (Fig. 3A). The magnitude of the inhibition was similar to that caused by E1A, suggesting a biologically relevant decrease. An NH_2-terminal truncation of expanded atrophin-1 similar to a fragment seen in a transgenic mouse model of DRPLA (*34*) altered transcription (Fig. 3B), whereas nontoxic atrophin-1 constructs had no effect. Thus, transcription inhibition correlates with polyglutamine toxicity for both huntingtin and atrophin-1.

Using a reporter construct containing three cyclic 3′,5′-adenosine monophosphate response element (CRE) sites, which uses endogenous CBP and endogenous CREB, we demonstrated similar and specific expanded repeat huntingtin transcription inhibition (Fig. 3C). Because CBP regulates other transcription factors such as ELK, we performed

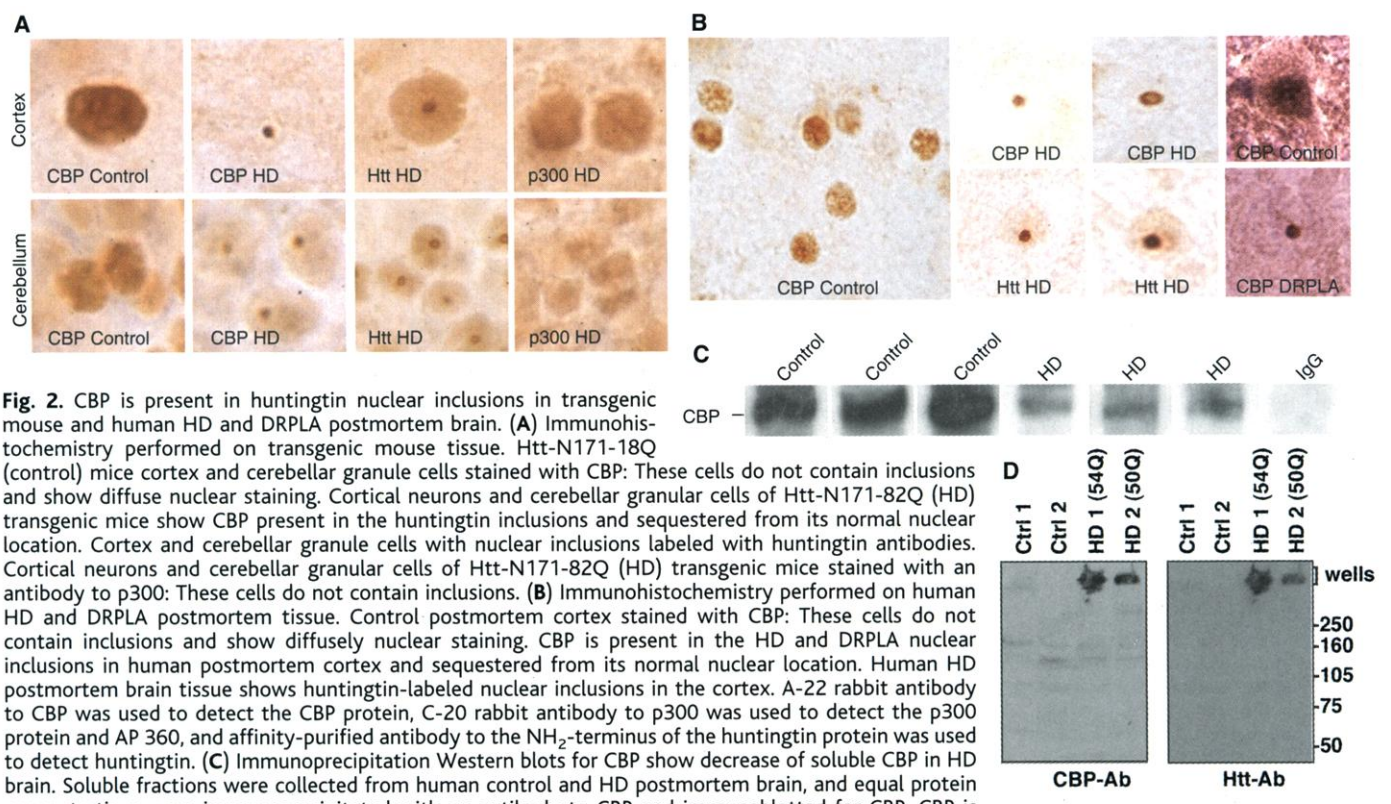

Fig. 2. CBP is present in huntingtin nuclear inclusions in transgenic mouse and human HD and DRPLA postmortem brain. (**A**) Immunohistochemistry performed on transgenic mouse tissue. Htt-N171-18Q (control) mice cortex and cerebellar granule cells stained with CBP: These cells do not contain inclusions and show diffuse nuclear staining. Cortical neurons and cerebellar granular cells of Htt-N171-82Q (HD) transgenic mice show CBP present in the huntingtin inclusions and sequestered from its normal nuclear location. Cortex and cerebellar granule cells with nuclear inclusions labeled with huntingtin antibodies. Cortical neurons and cerebellar granular cells of Htt-N171-82Q (HD) transgenic mice stained with an antibody to p300: These cells do not contain inclusions. (**B**) Immunohistochemistry performed on human HD and DRPLA postmortem tissue. Control postmortem cortex stained with CBP: These cells do not contain inclusions and show diffusely nuclear staining. CBP is present in the HD and DRPLA nuclear inclusions in human postmortem cortex and sequestered from its normal nuclear location. Human HD postmortem brain tissue shows huntingtin-labeled nuclear inclusions in the cortex. A-22 rabbit antibody to CBP was used to detect the CBP protein, C-20 rabbit antibody to p300 was used to detect the p300 protein and AP 360, and affinity-purified antibody to the NH_2-terminus of the huntingtin protein was used to detect huntingtin. (**C**) Immunoprecipitation Western blots for CBP show decrease of soluble CBP in HD brain. Soluble fractions were collected from human control and HD postmortem brain, and equal protein concentrations were immunoprecipitated with an antibody to CBP and immunoblotted for CBP. CBP is greatly reduced from the soluble fraction in HD tissue compared with controls. By contrast, immunoprecipitation Western blots with an antibody to β-tubulin showed no differences in β-tubulin. (**D**) CBP is sequestered into huntingtin aggregates in HD postmortem brain. Triton-insoluble fractions were collected from human control and HD postmortem brain, resuspended in 2% SDS, and analyzed by immunoblotting. In samples from HD but not control, aggregated protein trapped at the top of the wells contained huntingtin. When the blot was stripped and reprobed, CBP was also detected in the aggregates.

the transcription assay using a GAL4-ELK reporter construct and determined that only mutant huntingtin significantly inhibited transcription (Fig. 3D). To demonstrate specificity and determine that the decrease in transcription was not secondary to cellular toxicity caused by mutant huntingtin, we examined the effect of huntingtin on a reporter construct driven by p300-mediated transcription. Huntingtin with the expanded repeat had no effect on p300-mediated transcriptional activity (Fig. 3E). Similar lack of inhibition was found with a reporter construct that uses endogenous Sp1 (Fig. 3F). To determine whether the inhibition of CBP-mediated transcription was dependent on the presence of the polyglutamine in CBP, we performed the transcription assay using a Gal4-CBPΔQ reporter construct. Htt-N63-148Q reduced Gal4-CBP–mediated transcription, but not Gal4-CBPΔQ–mediated transcription (Fig. 3G). Thus, huntingtin specifically interferes with CBP-mediated transcription through polyglutamine interactions.

We hypothesized that huntingtin's interaction with CBP and sequestration from its normal site of action leads to cellular toxicity. Previous experiments in our laboratories demonstrated that the NH_2-terminal fragment of huntingtin with an expanded repeat is toxic to cells in culture (*14*, *35*). If our hypothesis is correct, then overexpression of CBP or CBPΔQ should rescue huntingtin toxicity (*36*). Figure 4A demonstrates that overexpression of CBP or especially of CBPΔQ abrogated the toxic effect of huntingtin (even though CBP or CBPΔQ alone had no significant effect on cell viability). Furthermore, overexpression of CBP or CBPΔQ also rescued cells from atrophin-1 toxicity (Fig. 4B). CBP's abrogation of toxicity is not due to a change in the level of expression of huntingtin or atrophin-1. Western blots showed similar intensities for each transfection condition, and the frequency and density of huntingtin aggregates were also similar. CBPΔQ was at least as effective as overexpression of normal full-length CBP (Fig. 4, A and B). By contrast, p300, Sp1, and truncated atrophin-1 with a normal polyglutamine repeat were ineffective at rescuing cellular toxicity (Fig. 4, C and D). Cellular toxicity experiments were also performed with rat primary cortical neurons. Neurons transfected with Htt-N63-18Q showed several long processes with green fluorescent protein (GFP) staining and normal nuclei with Hoechst 33342. Neurons

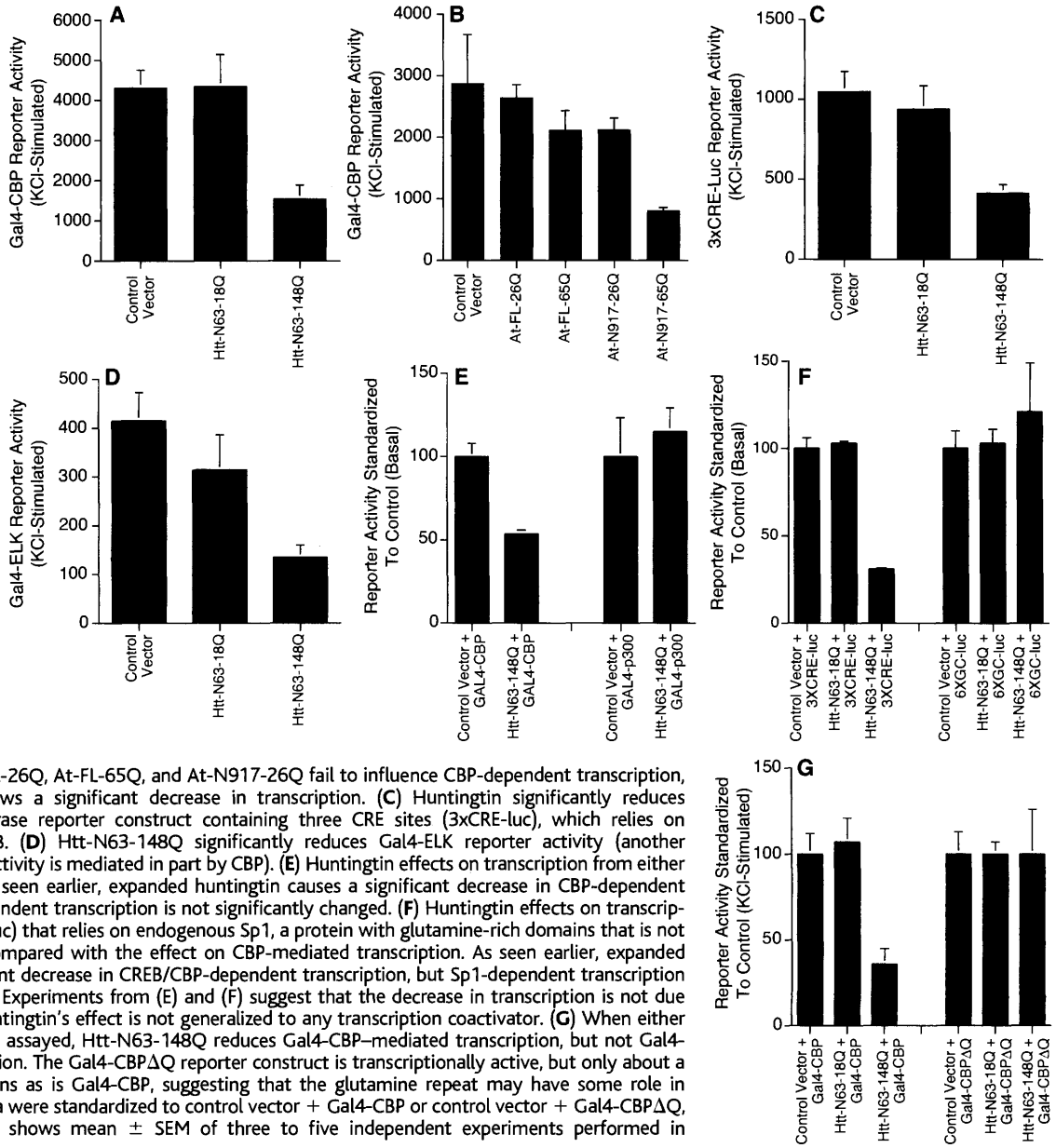

Fig. 3. Huntingtin or atrophin-1 with an expanded glutamine repeat decreases CBP-mediated transcription. Primary rat cortical neurons were transfected with luciferase reporter constructs and either control vector, Htt-N63-18Q, or Htt-N63-148Q for HD and either control vector, At-FL-26Q, At-FL-65Q, At-N917-26Q, or At-N917-65Q for DRPLA. Luciferase levels were measured for basal conditions or stimulated with 50 mM KCl for 12 hours. (**A**) Htt-N63-18Q shows transcriptional activation levels comparable to the control plasmid pCI. However, Htt-N63-148Q significantly decreases Gal 4-CBP transcription. These data indicate that expanded huntingtin interferes with CBP-mediated transcription. The graph shows KCl-stimulated transcription normalized to percent basal activity and mean ± SEM for at least three experiments. (**B**) Effects of atrophin on Gal4-UAS luciferase reporter activity. At-FL-26Q, At-FL-65Q, and At-N917-26Q fail to influence CBP-dependent transcription, whereas At-N917-65Q shows a significant decrease in transcription. (**C**) Huntingtin significantly reduces transcription, with a luciferase reporter construct containing three CRE sites (3xCRE-luc), which relies on endogenous CBP and CREB. (**D**) Htt-N63-148Q significantly reduces Gal4-ELK reporter activity (another transcription factor whose activity is mediated in part by CBP). (**E**) Huntingtin effects on transcription from either Gal4-CBP or Gal4-p300. As seen earlier, expanded huntingtin causes a significant decrease in CBP-dependent transcription, but p300-dependent transcription is not significantly changed. (**F**) Huntingtin effects on transcription from a GC box (6xGC-luc) that relies on endogenous Sp1, a protein with glutamine-rich domains that is not recruited into aggregates compared with the effect on CBP-mediated transcription. As seen earlier, expanded huntingtin causes a significant decrease in CREB/CBP-dependent transcription, but Sp1-dependent transcription is not significantly changed. Experiments from (E) and (F) suggest that the decrease in transcription is not due to death of neurons and huntingtin's effect is not generalized to any transcription coactivator. (**G**) When either Gal4-CBP or Gal4-CBPΔQ is assayed, Htt-N63-148Q reduces Gal4-CBP–mediated transcription, but not Gal4-CBPΔQ–mediated transcription. The Gal4-CBPΔQ reporter construct is transcriptionally active, but only about a third as functional in neurons as is Gal4-CBP, suggesting that the glutamine repeat may have some role in transcription activation. Data were standardized to control vector + Gal4-CBP or control vector + Gal4-CBPΔQ, respectively (G). All graphs shows mean ± SEM of three to five independent experiments performed in quadruplicates.

transfected with Htt-N63-148Q showed marked loss of processes by GFP staining and shrunken condensed nuclei by Hoechst 33342 staining, changes characteristic of degenerating neurons. Neurons cotransfected with Htt-N63-148Q and CBPΔQ demonstrated restored neuronal processes and normal nuclei (Fig. 4E). Quantification of GFP showed that CBPΔQ completely rescued huntingtin-mediated toxicity (Fig. 4F). These data suggest that interaction of expanded polyglutamine protein with the short polyglutamine tract in CBP specifically mediates cellular toxicity.

Our results suggest that expanded polyglutamine may exert toxic effects within cells by sequestering CBP, or other proteins containing short polyglutamine stretches, away from their critical sites of action. This effect depends on the expanded polyglutamine repeat within the disease protein and the short polyglutamine repeat in CBP. We suggest that there is a direct interaction between the two polyglutamine repeats, although it is possible that other proteins are also involved. A direct interaction could involve noncovalent "polar zippers" between the glutamine repeats (*15, 37*). Interactions between expanded polyglutamine tracts and short polyglutamine tracts in proteins such as CBP can provide an explanation for the polyglutamine disease "toxic gain of function" (*2*, *38*), because the effects on CBP localization, sequestration, and transcription are specific only to the expanded form of the polyglutamine disease protein. Other proteins within cells have short polyglutamine stretches and may also be involved. In this model, it is not the inclusions or aggregation that are directly toxic, but rather the indirect effect on other proteins such as CBP. Thus, our data suggest a unifying mechanism of cellular toxicity for glutamine repeat diseases.

Our data are also consistent with the emerging data linking transcriptional abnormalities with polyglutamine pathogenesis. McCampbell *et al.* recently showed that the androgen receptor with an expanded polyglutamine repeat, which is the cause of spinal and bulbar muscular atrophy (SBMA), interacts with CBP, leading to cell toxicity (*22*). It has also recently been shown that atrophin-1 and other polyglutamine proteins associate with $TAF_{II}130$ (*39*) and can interfere with CREB-mediated transcription. Furthermore, Steffan *et al.* reported that expanded huntingtin repressed transcription of a p53 reporter construct potentially through an interaction with the coactivator CBP (*23*). In a PC12 neuronal cell model with inducible expression of mutant huntingtin, we found decreases of CBP-associ-

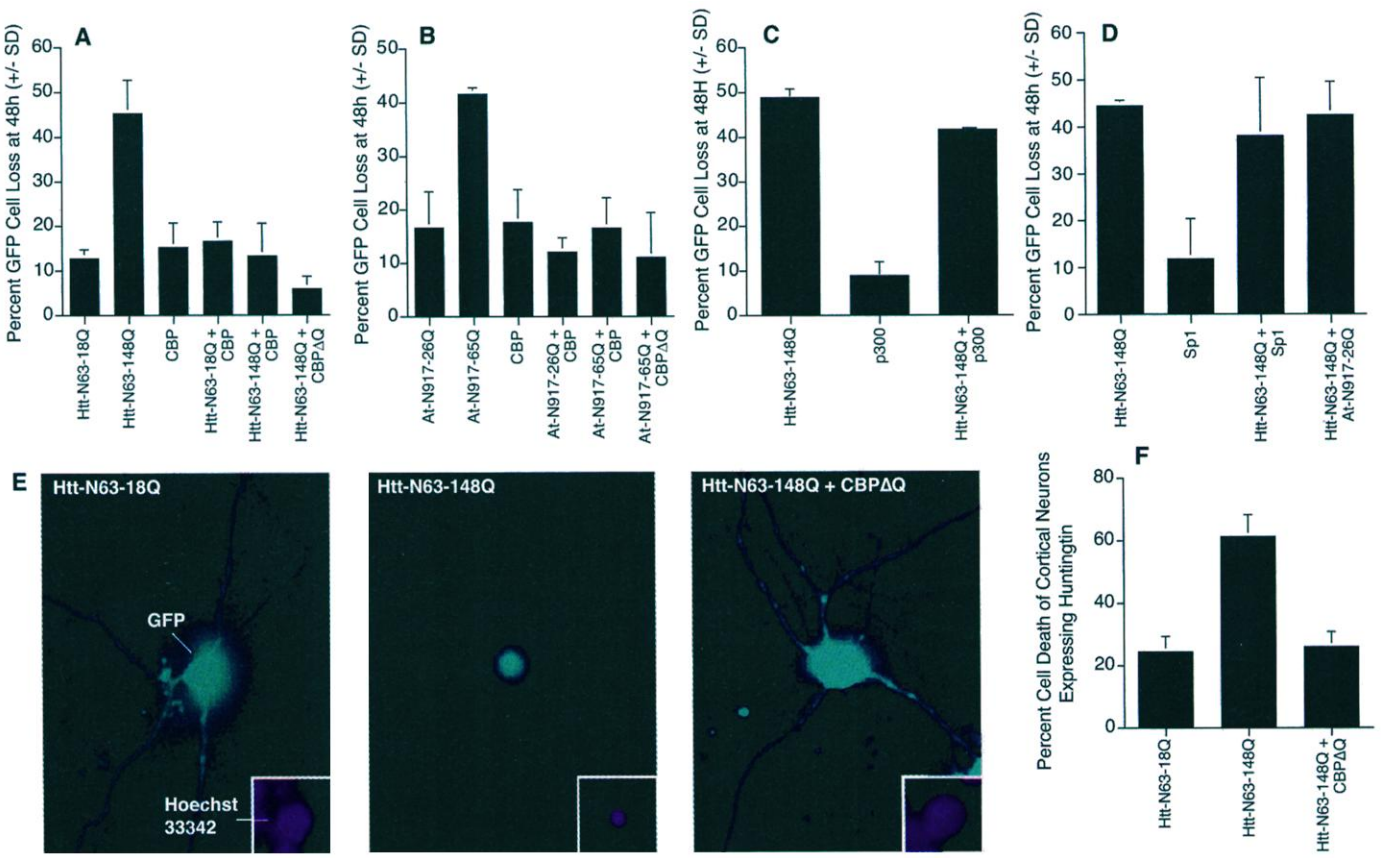

Fig. 4. CBP overexpression rescues cells from toxicity mediated by huntingtin or atrophin-1 with expanded polyglutamine repeats. N2a cells were cotransfected with GFP and an HD or a DRPLA construct alone or with CBP. Cell loss was quantified by counting GFP positive cells 48 hours later. (**A**) Htt-N63-148Q caused increased toxicity compared with the construct with 18 repeats or CBP alone. When excess CBP or CBPΔQ was cotransfected with Htt-N63-148Q, cell toxicity was reduced to baseline. (**B**) At-N917-65Q showed increased toxicity compared with the same construct with 26 repeats or CBP alone. When excess CBP or CBPΔQ was cotransfected with At-N917-65Q, cell toxicity was reduced to baseline. (**C**) When the homologous protein p300 containing a six glutamine stretch was cotransfected with Htt-N63-148Q, cell toxicity remained comparable to Htt-N63-148Q alone, indicating specificity. p300 was expressed, as determined by immunofluorescence or Western blot and determined to be functional by potassium depolarization. (**D**) When the glutamine-rich protein Sp1 was cotransfected with Htt-N63-148Q, cell toxicity remained comparable to Htt-N63-148Q alone, again demonstrating specificity. Also, At-N917-26Q, which is recruited into huntingtin aggregates (see Fig. 1), did not rescue cell toxicity, indicating specificity. All graphs are the average of at least three assays in triplicate standardized to LacZ and show standard deviations. (**E**) Cellular toxicity in cortical neurons. Cortical neurons were transfected with GFP and Htt-N63-18Q or Htt-N63-148Q alone or with GFP plus Htt-N63-148Q plus CBPΔQ. Cellular morphology with GFP or Hoechst 33342 demonstrated loss of processes and shrunken nuclei for huntingtin-induced toxicity, rescued by CBPΔQ. (**F**) The percentage of transfected cells that are nonviable compared with viable are shown. CBPΔQ rescued huntingtin-mediated cellular toxicity in cortical neurons. In all experiments, the amount of DNA transfected was kept constant by cotransfecting appropriate amounts of LacZ DNA when relevant.

ated histone acetyl transferase activity, as well as total histone acetylation at an early time point (*40*). Alteration of CBP-regulated gene expression may mediate neuronal dysfunction and neuronal toxicity. Gene expression screens have shown that genes that are regulated by CBP, such as enkephalin and Jun, are downregulated in HD transgenic mice and HD postmortem brain tissue (*41*, *42*). It is likely that there is not one single downstream survival mediator, but rather a number of survival-related transcripts. For example, BDNF, a cell survival protein whose expression is regulated by CREB, has recently been shown to be downregulated in HD patient tissue (*43*). Our data suggest that expanded polyglutamine interactions with proteins containing short polyglutamine stretches are relevant for polyglutamine pathogenesis and therefore that these interactions might be targets for future therapeutics.

References and Notes

1. C. A. Ross, *Neuron* **15**, 493 (1995).
2. H. L. Paulson, K. H. Fischbeck, *Annu. Rev. Neurosci.* **19**, 79 (1996).
3. J. F. Gusella, M. E. MacDonald, *Annu. Rev. Med.* **47**, 201 (1996).
4. C. A. Ross *et al.*, *Medicine* **76**, 305 (1997).
5. E. Scherzinger *et al.*, *Cell* **90**, 549 (1997).
6. S. W. Davies *et al.*, *Cell* **90**, 537 (1997).
7. F. Persichetti *et al.*, *Neurobiol. Dis.* **6**, 364 (1999).
8. D. Martindale *et al.*, *Nature Genet.* **18**, 150 (1998).
9. C. C. Huang *et al.*, *Somat. Cell Mol. Genet.* **24**, 217 (1998).
10. M. DiFiglia *et al.*, *Science* **277**, 1990 (1997).
11. H. L. Paulson *et al.*, *Neuron* **19**, 333 (1997).
12. M. W. Becher *et al.*, *Neurobiol. Dis.* **4**, 387 (1998).
13. I. A. Klement *et al.*, *Cell* **95**, 41 (1998).
14. J. K. Cooper *et al.*, *Hum. Mol. Genet.* **7**, 783 (1998).
15. M. F. Perutz, *Trends Biochem. Sci.* **24**, 58 (1999).
16. A. Kazantsev, E. Preisinger, A. Dranovsky, D. Goldgaber, D. Housman, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11404 (1999).
17. E. Preisinger, B. M. Jordan, A. Kazantsev, D. Housman, *Philos. Trans. R. Soc. London B Blio. Sci.* **354**, 1029 (1999).
18. J. C. Chrivia *et al.*, *Nature* **365**, 855 (1993).
19. A. Bonni *et al.*, *Science* **286**, 1358 (1999).
20. A. Riccio, S. Ahn, C. M. Davenport, J. A. Blendy, D. D. Ginty, *Science* **286**, 2358 (1999).
21. M. R. Walton, I. Dragunow, *Trends Neurosci.* **23**, 48 (2000).
22. A. McCampbell *et al.*, *Human Mol. Genet.* **9**, 2197 (2000).
23. J. S. Steffan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6763 (2000).
24. M. F. Peters, C. A. Ross, *Neurosci. Lett.* **275**, 129 (1999).
25. Mouse Neuro-2a (N2a) cells were plated in one-well chamber slides (Nunc), cultured overnight, and transfected with 0.5 μg of DNA with Lipofectamine Plus (Life Technologies) as recommended by the manufacturer's protocol. Cells were fixed with methanol at –20°C for 10 min. Cells were rinsed three times in phosphate-buffered saline (PBS) and blocked for 1 hour in 5% normal goat serum. Mouse monoclonal c-myc antibody or rabbit polyclonal (Santa Cruz Biotechnology) c-myc antibody was used to detect the myc epitope at the NH_2-terminus of huntingtin protein. A-22 rabbit antibody to CBP (Santa Cruz Biotechnology) was used to detect the CBP protein. C-20 rabbit antibody to p300 was use to detect overexpressed p300 protein. Mouse monoclonal HA antibody (Boehringer Mannheim) was used to detect the HA epitope at the COOH-terminus of the Sp1 protein. Cells were incubated with antibody for 3 hours at 37°C. Washed cells were incubated with fluorescein isothiocyanate or Cy3-conjugated secondary antibody (Jackson Laboratories) diluted 1:250. TOTO-3 (Molecular Probes) was used as a nuclear marker. Cover slips were mounted with Vectashield (Vector Laboratories). Cells were viewed on a Noran confocal microscope. CBP labeling was judged to be specific because preincubating antibody with a CBP peptide eliminated staining. N2a cells were cotransfected with huntingtin and CBP or CBPΔQ and solubilized in PBS–2% NP-40. The soluble fraction was immunoprecipitated with a c-myc antibody that recognizes the tagged huntingtin constructs, antibody to CBP, or rabbit immunoglobulin G (IgG) and then Western blotted for CBP or c-myc. Equivalent levels of huntingtin were immunoprecipitated when antibody to myc was used to immunopreciptate huntingtin, and equivalent levels of CBP were immunoprecipitated when antibody to CBP was used to immunopreciptate CBP. CBP and CBPΔQ were expressed at equal levels by Western blot analysis.
26. G. Schilling *et al.*, *Hum. Mol. Genet.* **8**, 397 (1999).
27. L. Mangiarini *et al.*, *Cell* **87**, 493 (1996).
28. Transgenic mouse brains or human postmortem tissue were fixed with 4% paraformaldehyde. Cut sections were permeabilized in methanol:peroxide for 10 min. The tissue was washed and blocked in 5% normal goat serum for 45 min and incubated in primary antibody overnight. A peptide antibody made to the NH_2-terminus of huntingtin AP 360 was diluted 1:10,000 for transgenic mouse sections and 1:2000 in human postmortem brain sections. A-22 rabbit antibody to CBP (Santa Cruz Biotechnology) was used at 1:1000 for transgenic mice and 1:2000 for human postmortem tissue to detect the CBP protein. C-20 rabbit antibody to p300 (Santa Cruz Biotechnology) was used at 1:1000 for transgenic mice to detect the p300 protein. Washed tissue was incubated in secondary antibody diluted at 1:200 for 3 hours. Washed tissue was then incubated in ABC reagent Vectastain-Elite ABC kit (Vector Laboratories) diluted at 1:50 for 45 min and developed with DAB:peroxide for 5 min. CBP labeling was judged to be specific because preincubating antibody with peptide eliminated staining. Human cerebral cortex grades 2 and 3 according to Vonsattel classification from HD patients or controls were homogenized in 10 volumes of 50 mM tris (pH 7.5) and 1% triton. The homogenate was spun for 30 min at 150,000*g*. The supernatant was assayed for protein concentration, and equal amounts of protein were immunoprecipitated with A-22 rabbit antibody to CBP and protein A beads for 4 hours and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) immunoblot. The pellet was resuspended in 50 mM tris (pH 7.5) with 2% SDS and boiled for 4 min. Samples were spun at 1000*g* for 2 min before SDS-PAGE immunoblot analysis.
29. S. Chawla, G. E. Hardingham, D. R. Quinn, H. Bading, *Science* **281**, 1505 (1998).
30. S. Impey *et al.*, *Nature Neurosci.* **1**, 595 (1998).
31. G. E. Hardingham, S. Chawla, F. H. Cruzalegui, H. Bading, *Neuron* **22**, 789 (1999).
32. S. C. Hu, J. Chrivia, A. Ghosh, *Neuron* **22**, 799 (1999).
33. Primary neuronal cultures were prepared from rat embryonic cortex at the stage of embryonic day 16 (E16) and cultured according to standard procedures (*44*). After 5 days in vitro, cells were transfected with reporter plasmids and overexpression vectors as indicated along with 20 ng of CMV–β-Gal as internal control with the method of Ca^{2+}-phosphate precipitate coprecipitation (*45*). After 48 hours of culture, the cells were stimulated with 50 mM KCl and further incubated for 12 hours. Reporter gene activity was assayed by the chemiluminescence method (Promega). Transfection efficiencies were normalized by the value of the internal control of unstimulated wells. Experiments were carried out at least three times with three different wells, and data are presented as means ± SE. When control vector plus Gal4 alone was transfected, only 78 ± 2 reporter activity units were detected, and when Htt-N63-18Q or Htt-N63-148Q was transfected, 84 ± 4 and 63 ± 4 reporter activity units were detected, respectively, indicating that the reporter constructs have almost no activity and that huntingtin does not interfere with it. The 3XCRE-luc reporter gene was constructed by inserting three repeats of Ca/CRE response sequence (5′-GTTGACGTCAA-3′; consensus CRE site underlined) upstream of the minimum promoter of the thymidine kinase gene. CBP overexpression vector was a gift of R. H. Goodman (Oregon Health Science University). The poly-Q sequence (amino acids 2202 to 2216) in CBP was deleted from the CBP overexpression vector with polymerase chain reaction–based mutagenesis (*46*) and designated CBPΔQ. GAL4-CBP was a gift from H. Bading (Medical Research Council, UK). 5XGAL4-luc was a gift from R. A. Maurer (Oregon Health Science University). 6XGC-luc was a gift form J. E. Kudlow (University of Alabama). Sp1 overexpression vector (with HA tag) was a gift from E. Seto (University of South Florida).
34. G. Schilling *et al.*, *Neuron* **24**, 275 (1999).
35. M. F. Peters *et al.*, *Mol. Cell. Neurosci.* **14**, 121 (1999).
36. N2a cells were seeded in six-well plates and cotransfected with GFP and lacZ or an Htt-N63 construct plus lacZ or an Htt-N63 construct plus CBP. GFP-positive fluorescent cells were counted 48 hours later by capture on a Zeiss Axiovert 135 inverted microscope through IP Spectrum Labs image analysis software. Each experiment contains cells from 10 randomly selected fields from each of three wells. Mean and standard deviations are taken from at least three different experiments. As a measure of cell loss, cells were counted. We have previously shown that this measure of cell loss correlates with cell death as measured by propidium iodide to label dead cells and Hoechst 33342 to label total cells. Plasmids purified in separate preparations gave similar results. Primary neuronal cultures were prepared from rat embryonic cortex at the stage of E16 and cultured according to standard procedures (*44*). After 5 days in vitro, cells were cotransfected with GFP and an Htt-N63 construct plus lacz or an Htt-N63 construct plus CBPΔQ. Cell death was quantitated at 72 hours on the basis of cell number and morphology with GFP (like β-Gal has been used) as a marker for soma and processes (*47*, *48*). Cells were counted as viable if they had several extensive processes and counted as nonviable if they had shrunken cell bodies and little or no processes. Two experiments performed in quadruplicate demonstrated similar results.
37. M. F. Perutz, T. Johnson, M. Suzuki, J. T. Finch, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5355 (1994).
38. D. Housman, *Nature Genet.* **10**, 3 (1995).
39. T. Shimohata *et al.*, *Nature Genet.* **26**, 29 (2000).
40. S. Igarashi *et al.*, *Soc Neurosci. Abstr.* **382.7**, 1030 (2000).
41. R. Luthi-Carter *et al.*, *Hum. Mol. Genet.* **9**, 1259 (2000).
42. E. K. Richfield *et al.*, *Ann. Neurol.* **37**, 335 (1995).
43. I. Ferrer *et al.*, *Brain Res.* **866**, 257 (2000).
44. M. Gonzalez-Zulueta *et al.*, *J. Neurosci.* **18**, 2040 (1998).
45. X. Tao, S. Finkbeiner, D. B. Arnold, A. J. Shaywitz, M. E. Greenberg, *Neuron* **20**, 709 (1998).
46. S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* **77**, 51 (1989).
47. A. Ghosh, M. E. Greenberg, *Neuron* **15**, 89 (1995).
48. R. Threadgill *et al.*, *Neuron* **19**, 625 (1997).

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